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Original Article

Protective effects of strawberry and mulberry fruit polysaccharides on inflammation and apoptosis in murine primary splenocytes

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ABSTRACT

This study isolated polysaccharides from strawberry (SP) and mulberry (MP) fruit juice to compare their cytokine secretion regulatory and antiapoptotic activities using murine primary splenocytes. SP and MP in the absence or presence of lipopolysaccharide (LPS) were administered to splenocytes for 48 hours. The culture supernatant was used for cytokine secretion assay using the enzyme-linked immunosorbent assay method. The cell pellet was used for the determination of anti-/proapoptotic protein (B cell lymphoma 2/Bak) levels in the cells using the Western blotting method. The results showed that SP and MP treatment at appropriate concentrations significantly increased the proliferation of splenocytes ($p < 0.05$). SP and MP treatments in the absence of LPS, and SP treatments in the presence of LPS significantly decreased T helper type 1/T helper type 2 ($p < 0.05$), and SP in the presence of LPS slightly decreased tumor necrosis factor-α/interleukin-10 (pro-/anti-inflammatory) cytokine secretion ratios by splenocytes, suggesting that SP has strong and MP has mild anti-inflammation potential via modulating cytokine secretion profiles. However, MP treatment at an appropriate concentration in the absence of LPS exhibited an antiapoptotic activity via modulating pro- (Bak) and antiapoptotic (B cell lymphoma 2) protein expression ratios, suggesting that MP may protect primary immune cells from apoptotic cell death. Overall, our findings suggest that SP has better anti-inflammation potential, whereas MP has better cell proliferation and antiapoptotic potential *in vitro*.

1. Introduction

T lymphocytes, T helper type 1 (Th1) and Th2 lymphocytes produce some common cytokine products; others are mutually exclusive [1]. Th1 cells produce interferon (IFN)-γ, tumor necrosis factor (TNF)-α/β, interleukin (IL)-2, IL-3, IL-10, and granulocyte macrophage colony-stimulating factor (GM-CSF). Th2 lymphocytes synthesize IL-3, IL-4, IL-5, IL-10, and GM-CSF [1]. Among cytokines, IL-1, TNF, and IL-6 can generally be classified as proinflammatory cytokines because they highlight the way and initiate local inflammation within injured

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tissues [2]. In contrast to proinflammatory cytokines, IL-10 is recognized as an anti-inflammatory cytokine because it is produced by Th2 cells, T regulatory cells (Th3 cells), macrophages, and some B cells to inhibit the synthesis of other cytokines and macrophage functions during the late inflammation phase [3]. In general, Th1 cell secreted cytokines enhance inflammatory responses *in vivo*; however, Th2 cell secreted cytokines that potentially inhibit the secretion of Th1 cells are recognized as anti-inflammatory cytokines. Appropriate regulation of pro- and anti-inflammatory and Th1/Th2 cytokine expression in immune cells by potential food components may avoid immune disorder diseases.

Apoptosis, programmed cell death is a complicated process by which cells undergo a form of non-necrotic cellular suicide involving the immuno-pharmaco-redox signaling pathways [4]. B cell lymphoma 2 (Bcl-2) family members comprise three subgroups: the antiapoptotic family members (Bcl-2, Bcl-xL, Mcl-1, A1, Bcl-b, and Bcl-w), the proapoptotic BH3-only proteins (Bid, Bad, Bmf, Bik, Noxa, Puma, and Hrk), and the proapoptotic Bax/Bak subfamily play an important role in apoptosis progression in different cell types, especially in lymphocytes [5,6]. Bax, that is Bcl-2-associated X protein, and Bak, a Bcl-2 homologous antagonist/killer, are critical in programmed cell death regulation. Healthy cells have high relative amounts of free antiapoptotic Bcl-2 family members to bind and sequester proapoptotic Bax and Bak [5]; freeing Bax and Bak, a requisite gateway to mitochondrial dysfunction and cell death in response to diverse stimuli, enhance mitochondrial cytochrome *c* release and induce caspase cascade activation [7]. In contrast to healthy cells, BH3-only proteins are activated under cellular stress, via transcriptional regulation or post-translational modification, and then combined with the anti-apoptotic Bcl-2 family members [5]. Bax and Bak initiate apoptosis in higher eukaryotes [8], and play an essential role in B cell homeostasis and the prevention of autoimmune disease [9]. Based on the changes in antiapoptotic Bcl-2 and proapoptotic Bak protein levels, apoptotic status in cells may be delineated. Apoptosis, that is, spontaneously programmed cell death resulting from internal or external stimuli is generally regarded as harmless; however, uncontrolled or unexpected apoptotic cell death is involved in many degenerative diseases such as neurodegenerative diseases, including Alzheimer's and Parkinson's [10], and neuromuscular diseases [11]. Therefore, antiapoptotic therapy in normal cells may be beneficial for the corresponding human diseases.

Recently, non-starch polysaccharides have been the focus of much attention for their potential physiological effects. It was found that *Typha latifolia* L. fruit polysaccharides induced differentiation and stimulated the proliferation of human keratinocytes *in vitro* [12]. Polysaccharides from the leaves, roots, and fruits of *Panax ginseng* C.A. Meyer are reported to have different biological activities, such as antitumor, immunoregulatory, anti-adhesive, antioxidant, antiulcer, anti-radiation, antisepticemic, hepatoprotective, anti-asthmatic, antidepressant, qi-invigorating, antifatigue, and antiviral activity [13]. Most recently, we found that polysaccharides isolated from strawberry and mulberry juice modulated Bak and Bcl-2 protein levels in murine primary macrophages, suggesting that the polysaccharides protected lipopolysaccharide (LPS)-stimulated macrophages from apoptotic cell death. Furthermore, a negative correlation between cytokine secretion levels and Bcl-2 protein levels suggested that proinflammatory IL-1β and IL-6 cytokines decreased Bcl-2 levels in the LPS-stimulated macrophages [14]. Polysaccharides from plant foods may show promise for their immunomodulatory activities, such as antiapoptosis and anti-inflammation.

Polysaccharides from mulberry and strawberry fruits on immunomodulation are still scarcely reported. The spleen is the largest immune organ and the splenocytes *in vitro* may reflect systemic immune status in the body. To determine the anti-inflammatory and antiapoptotic effects of strawberry and mulberry polysaccharides, primary splenocytes from female BALB/c mice treated with the fruit polysaccharides in the absence or presence of LPS were cultured. Both Th1/Th2 cytokine secretion profiles and Bcl-2/Bak (anti-/proapoptotic) protein levels in the splenocytes were determined using enzyme-linked immunosorbent assay (ELISA) and Western blotting protein assay to compare their anti-inflammatory and antiapoptotic activities of different fruit polysaccharides.

2. Materials and methods

2.1. *Isolation of strawberry and mulberry fruit polysaccharides*

Strawberry (*Fragaria ananassa*) and mulberry (*Morus alba*) fruits were purchased from a local supermarket in Taichung, Taiwan. The edible portions were weighed, washed, and chopped to squeeze fruit juice using a manual stainless screw squeezer (Vegetable and Fruit Grinder, manual type, Mei-Er-Then Co., Ltd, Taipei, Taiwan, R.O.C.). The juice was centrifuged at 10,000*g* (4°C) for 30 minutes, and then the supernatant was collected using suction filtration through filter papers (Toyo No. 5B). The filtrate was measured, lyophilized, and stored at −30°C for future use [15]. Polysaccharides are ethanol-insoluble compounds. To isolate polysaccharides, an aliquot of 1 g of the lyophilized fruit juice was added with 12 mL of deionized water to dissolve the juice sample. The juice was added with three volumes of 95% ethyl alcohol to achieve a mixture with a final concentration of 70% ethyl alcohol [16]. The mixture was allowed to stand with slow stirring at 4°C for 48 hours to precipitate ethanol-insoluble polysaccharides. The resultant mixture was centrifuged at room temperature, 5500*g* for 20 minutes to isolate ethanol-insoluble polysaccharides. The supernatant was removed, and then the residue was washed twice with the ethanol. The insoluble pellets (polysaccharides) were isolated and the solvent removed using nitrogen gas, lyophilized, and stored at −30°C until use. The yields of isolated strawberry fruit polysaccharide (SP) and mulberry fruit polysaccharide (MP) from the lyophilized powder of strawberry and mulberry fruit juice were 14.0 ± 6.3% and 10.6 ± 0.7%, respectively. The protein and carbohydrate contents of the isolated polysaccharide samples were further analyzed. The protein contents of the polysaccharide samples were analyzed using the BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instruction, using a 96-well microtiter plate [3]. The carbohydrate content in the SP and MP samples was analyzed using the

phenol–sulfuric acid method; the basic protocol of Dubois et al [17] was followed, with micro-plate format modifications [18]. The ratios between carbohydrate and protein contents in SP and MP were 43%:57% (w/w) and 28.4%:71.6% (w/w), respectively. The maximum absorption peak of SP and MP appeared at 240 nm with a small shoulder around 280–310 nm, suggesting that SP and MP might be glycoproteins [14]. To confirm whether SP or MP was contaminated with endotoxin LPS, polymyxin B which neutralizes LPS effects by high affinity to lipid A of LPS was added to SP-, MP-, or LPS-stimulated splenocyte cultures, respectively. Our preliminary data indicated that LPS did not contaminate polysaccharides in the isolation procedure [16].

2.2. Source of murine primary splenocytes

Female BALB/cByJNarl mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan, R.O.C. and were maintained in the Department of Food Science and Biotechnology at the National Chung Hsing University, Taichung, Taiwan, R.O.C. The mice were housed and kept on a chow diet (laboratory standard diet, Diet MF 18, Oriental Yeast Co., Ltd, Osaka, Japan). The animal room was kept on a 12-hour light and 12-hour dark cycle. Constant temperature (25 ± 2°C) and relative humidity (50–75%) were maintained. After the mice were acclimatized for 4 weeks, they were sacrificed to obtain splenocytes. BALB/c strain mice weighting 20–25 g were used throughout the experiment. The animal use protocol for these experiments was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National Chung Hsing University, Taiwan, R.O.C. The primary splenocyte cells from mice were collected according to the method described by Lin and Tang [19]. Briefly, the adult female BALB/c mice were anesthetized with diethyl ether, bled using a retro-orbital venous plexus puncture to collect blood, and immediately euthanized by CO_2 inhalation. The splenocytes were prepared by aseptically removing the spleens from the BALB/c mice. The spleens were homogenized in tissue culture medium (TCM, a serum replacement; Celox Laboratories Inc., Lake Zurich, IL, USA), suspended in a medium consisting of 10 mL TCM, 500 mL RPMI 1640 medium (Atlanta Biologicals Inc., Norcross, GA, USA), and 2.5 mL antibiotic–antimycotic solution (100 × PSA) containing 10,000 U/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B in 0.85% saline (Atlanta Biologicals Inc., Norcross, GA, USA) with the aid of a syringe piston. Single spleen cells were collected and treated by lysing the red blood cells with RBC lysis buffer [0.017 M Trizma base (Sigma-Aldrich Co., St Louis, MO, USA), 0.144 M ammonium chloride (Sigma-Aldrich Co.), pH 7.4, 0.20 μm filtered]. Splenocytes were isolated from each animal and adjusted to a concentration of 1×10^7 cells/mL in TCM medium with a hemocytometer using the trypan blue dye exclusion method.

2.3. Determination of optimal treatment SP and MP concentrations for murine primary splenocytes

To test the SP and MP treatment effects on the cell viability of murine primary splenocytes, possible cytotoxicities of SP (0 mg/mL, 0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, and 4.0 mg/mL) and MP (0 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, and 4.0 mg/mL) on murine splenocytes administered at various concentrations for 48 hours were determined. LPS (L-2654, Sigma-Aldrich Co.) at 5 μg/mL was also selected as a control. The cell viability at the end of each experiment was determined by the 3-(4,5-dimethylthiazol-2,5-diphenyl)-tetrazolium bromide (MTT, Sigma-Aldrich Co.) assay [19,20]. Briefly, after incubation of the cells cultured in the plate, aliquots of 10 μL (5 mg/mL) MTT in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.22 μm filtered) were added to each well for another 4 hours. The plates were centrifuged at 400*g* for 10 minutes to discard the media. The plates were carefully washed with PBS buffer three times to avoid possible interference with the MTT formazan crystals in cells due to sample colors. Aliquots of 100 μL dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) were added to each well and oscillated for 30 minutes to lyse the cell membrane and release the MTT formazan from cells. The absorbance was measured at 550 nm on a plate reader (ELISA reader, ASYS Hitech, GmbH, Eugendorf, Austria). The cell viability was directly expressed as absorbance at 550 nm. Optimal treatments of SP and MP concentrations were determined based on the cell viability (A_{550}) compared to the control.

2.4. Cultures of murine splenocytes with optimal SP and MP concentrations

The splenocytes in the absence or presence of stimulus LPS (5 μg/mL) and optimal concentrations of SP (0 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL) or MP (0 μg/mL, 250 μg/mL, 750 μg/mL, and 1250 μg/mL) were co-plated in 48-well plates. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for up to 48 hours. The plates were then centrifuged at 400*g* for 10 minutes to obtain cell culture supernatants and pellets for cytokine secretion assay and Western blotting assay, respectively.

2.5. Measurement of Th1/Th2 and proinflammatory and anti-inflammatory cytokine levels secreted by splenocyte cultures using ELISA

The culture supernatants of six biological replicates in each individual treatment were collected to measure cytokine levels using sandwich ELISA kits, respectively. The concentrations of cytokines including IL-2, IL-4, IL-5, IL-10, IL-12, and TNF-α were assayed according to the cytokine ELISA protocol in the manufacturer's instructions (mouse DuoSet ELISA Development system, R&D Systems, Minneapolis, MN, USA). The sensitivity of these cytokine assays was 15.6 pg/mL.

2.6. Determination of Bcl-2/Bak protein levels in murine primary splenocytes treated with SP and MP using the Western blotting assay method

The collected cell pellets were lysed and shaken at 4°C for 10 minutes with 200 μL of an extraction buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM sodium orthovanadate, 100 μg/mL phenylmethylsulfonyl

fluoride (PMSF), 0.225 U/mL aprotinin, and 0.1% sodium dodecyl sulfate (SDS) [21]. The mixture was allowed to stand at 4°C for an additional 30 minutes. The resulting mixtures were collected into an eppendorf, ultra-sonicated on ice with a sonicator (Misonix S-3000, Misonix Inc., Farmingdale, NY, USA) for 1 minute (10 seconds × 6), and centrifuged at 4°C, 16,000*g* for 20 minutes. The supernatants (intracellular proteins) were collected and added with another 12 mL phosphate buffer (pH 7.0, 75 mM). The protein concentration was determined using the Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). The protein concentration from each individual treatment was adjusted to the same (~1 mg/mL) using the lysis buffer. The intracellular proteins were obtained and stored at −80°C until use. Aliquots of 10 μL (~10 μg of protein) from each cell lysate were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% polyacrylamide gel containing 0.1% SDS using a Mini-Protein II cell (Bio-Rad Laboratories, Inc., Berkeley, CA, USA) and further subjected to Western blotting assay [14]. The molecular weights of the Bcl-2, Bak, and β-Actin proteins are ~26 kDa, ~30 kDa, and ~43 kDa, respectively. The level of β-Actin (a stable expression housekeeping protein) was selected to compare and calibrate the possible difference of total protein level loaded in each individual treatment. Bcl-2 and Bak protein expression levels were normalized against β-Actin. Data were expressed as arbitrary units based on densitometry scanning on the same membrane.

2.7. Statistical analysis

Data were analyzed using the Windows SAS program (Version 8.0, SAS Institute Inc., Cary, NC, USA). Data were expressed as mean ± standard error of the mean (SEM) using analysis of variance (ANOVA), if justified by the statistical probability ($p < 0.05$), followed by Duncan's new multiple range test or Dunnett's *t* test. Differences were considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1. SP and MP treatment effects on murine primary splenocyte cell viability

The aim of this study was to compare the effects of isolated polysaccharides from strawberry (SP) and mulberry (MP) fruit juice on cytokine secretion profiles and antiapoptotic activities using murine primary splenocytes. To avoid excessive cytotoxicity at high concentrations, appropriate concentrations of SP and MP were used to assess their effects on the cell viability of primary splenocytes. The remaining cell viability was determined by the MTT assay, showing that SP treatment does not have cytotoxicity on the splenocytes even at 4.0 mg/mL (Fig. 1A), and MP treatment lower than 2.0 mg/mL did not have cytotoxicity on the splenocytes (Fig. 1B). Interestingly, SP treatment significantly increased cell proliferation of primary splenocytes ($p < 0.05$). Mouse primary splenocytes are composed of 41.54% B cells and 47.11% T cells [22]. The other immune cells in splenocytes are presumed macrophages and dendritic cells that are not stimulated to proliferate *in vitro*. Thus, the proliferating cells in splenocytes *in vitro* are supposed to be B cell and/or T cells. LPS, a B cell mitogen, also significantly increased cell proliferation of primary splenocytes ($p < 0.05$; Fig. 1). Our results suggested that SP treatment higher than 0.125 mg/mL might stimulate B cell and/or T cell proliferation. Importantly, MP treatment significantly increased primary splenocyte cell proliferation ($p < 0.05$; Fig. 1B). Similar to SP, MP treatment at the range from 0.125 mg/mL to 2 mg/mL suggested stimulation of B cell and/or T cell proliferation [22]. A novel purified protein from *Amaranthus spinosus* water extract exhibited immunostimulating activity via directly stimulating B lymphocyte activation *in vitro* [22]. This study further suggested that polysaccharides, possibly glycoproteins, from strawberry and mulberry fruit juice possessed immunomodulatory activities via stimulating the proliferation of splenocytes (Fig. 1).

SP has an obviously lower toxicity on splenocytes than MP, although both SP and MP have extremely low cytotoxicity. However, MP has much higher immunostimulating activity on splenocyte proliferation than SP at their noncytotoxic concentrations. To avoid unpredictable cytotoxicities at high concentrations, appropriate concentrations of SP (0 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL) and MP (0 μg/mL, 250 μg/mL, 750 μg/mL, and 1250 μg/mL) in the absence or presence of LPS (5 μg/mL) were used for the following splenocyte experiments.

3.2. SP and MP treatment effects on cytokine secretion by murine primary splenocytes in the absence or presence of LPS

To unravel the effects of SP and MP treatments on Th1/Th2 and pro-/anti-inflammatory cytokine secretions, SP and MP at the indicated noncytotoxic concentrations were added to splenocyte cultures for 48 hours in the absence or presence of LPS. Table 1 shows the effects of SP on Th1/Th2 and pro-/anti-inflammatory cytokine secretion in the absence of LPS. The results showed that IL-10 (Th2), IL-12 (Th1), and TNF-α (Th1) cytokine levels secreted by the SP-treated splenocytes significantly increased in a concentration-dependent manner ($p < 0.05$). However, IL-2 (Th1) cytokine levels slightly decreased by SP in a concentration-dependent manner. Both IL-2 and IFN-γ are typical Th1 cytokines. However, we found that IFN-γ levels secreted by splenocytes in these experiments were too low to be detectable. Therefore, we selected the IL-2/IL-10 ratio as a common Th1/Th2 activity index. Importantly, IL-2/IL-10 (Th1/Th2) cytokine secretion ratios were significantly decreased by SP in a concentration-dependent manner ($p < 0.05$). Furthermore, IL-10 is a cytokine synthesis inhibitory factor that may play a role for anti-inflammation. Our results suggested that SP has Th2 inclination and anti-inflammation potential in the absence of LPS. Table 2 shows the effects of SP on Th1/Th2 or pro-/anti-inflammatory cytokine secretion in the presence of LPS. The results showed that TNF-α (a Th1 and proinflammatory cytokine) levels secreted by the SP-treated splenocytes significantly increased ($p < 0.05$). IL-10 (a Th2 and anti-inflammatory cytokine) levels were also increased by SP in a concentration-dependent manner. However, IL-2 (Th1) and IL-12 (Th1) cytokine levels were not significantly influenced by SP. Importantly, IL-2/IL-10 (Th1/

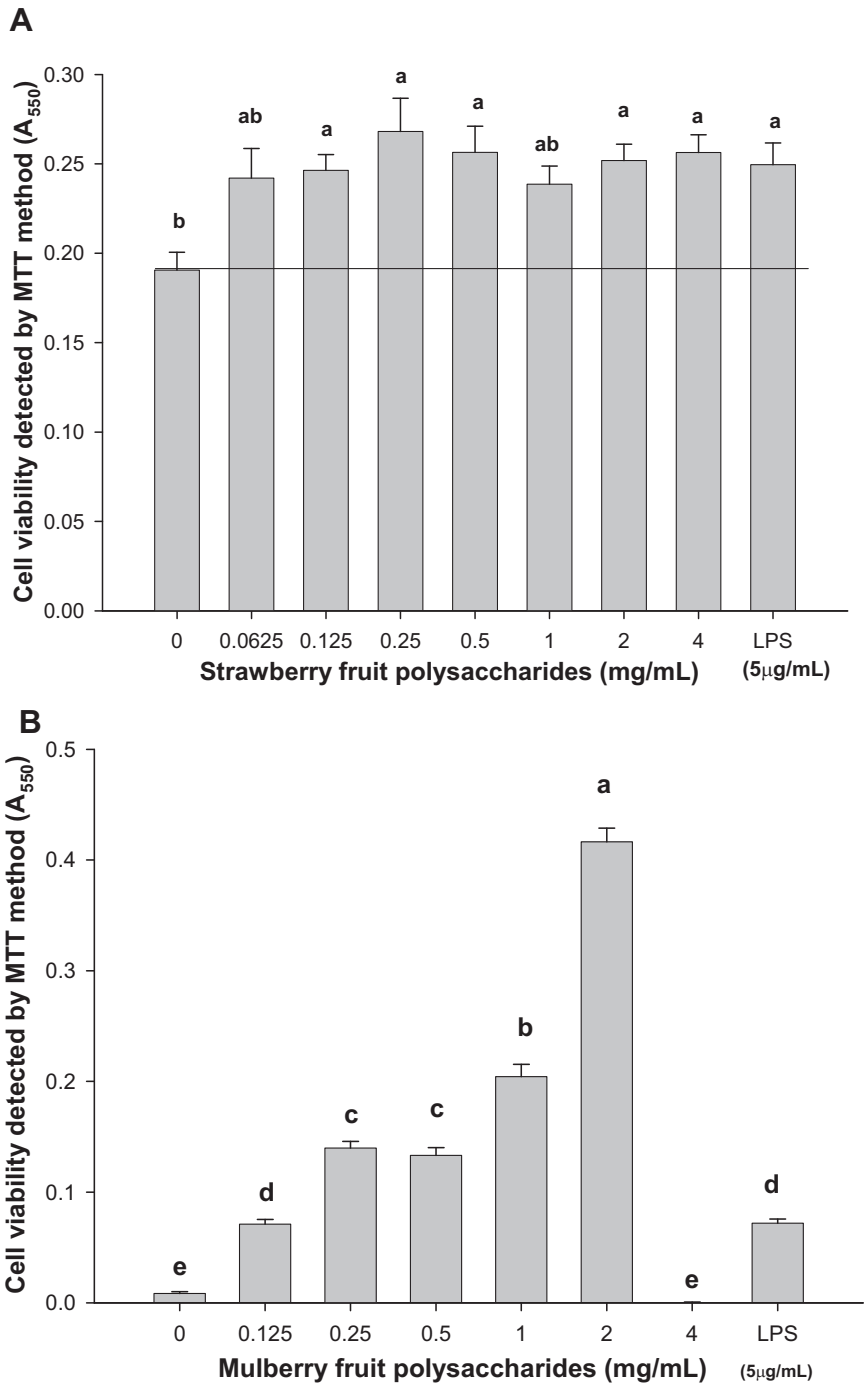

Fig. 1 – Strawberry (A) and mulberry (B) fruit polysaccharide effects on the viabilities of splenocytes from BALB/c mice. Data are mean ± SEM ($n = 6$ biological determinations). Bars not sharing the same letters are significantly different from each other ($p < 0.05$), assayed by one-way analysis of variance, followed by Duncan's multiple range test. Original cell density was 5×10^6 cells/mL. The absorbance (A_{550}) of each mulberry fruit polysaccharide treatment has been subtracted sample background to avoid the color disturbance of mulberry fruit polysaccharides.

Th2) cytokine secretion ratios were significantly decreased by SP in a concentration-dependent manner ($p < 0.05$), but TNF-α/IL-10 (pro-/anti-inflammatory) cytokine secretion ratios were just slightly decreased by SP ($p > 0.05$). Our results suggested that SP has strong Th2 inclination and mild anti-inflammation potential in the presence of LPS.

Table 3 shows the effects of MP on Th1/Th2 and pro-/anti-inflammatory cytokine secretion in the absence of LPS. The results showed that IL-4, IL-5, IL-10, IL-12, and TNF-α levels secreted by the MP-treated splenocytes significantly increased in a concentration-dependent manner ($p < 0.05$). IL-2 also significantly increased by MP ($p < 0.05$). Importantly, (IL-

Table 1 – Effects of strawberry fruit polysaccharide (SP) on cytokine secretion by murine primary splenocytes.

Cytokine levels (pg/mL)[a,b,c,d,e]	SP (μg/mL)			
	0	250	500	1000
IL-2	27 ± 4	27 ± 5	25 ± 3	19 ± 2
IL-4	n.d.	n.d.	n.d.	n.d.
IL-5	n.d.	n.d.	n.d.	n.d.
IL-10	9 ± 7^{B}	187 ± 53^{A}	239 ± 46^{A}	291 ± 29^{A}
IL-12	7 ± 2^{C}	8 ± 3^{BC}	18 ± 2^{AB}	25 ± 1^{A}
TNF-α	179 ± 80^{B}	717 ± 29^{A}	763 ± 41^{A}	770 ± 62^{A}
IL-2/IL-10 (pg/pg)	3.00 ± 1.83^{A}	0.15 ± 0.05^{B}	0.13 ± 0.02^{B}	0.07 ± 0.01^{B}

[a] Data are mean ± SEM ($n = 6$ biological determinations).
[b] The cell density was 5×10^6 cells/mL.
[c] Data within the same row not sharing a common superscript capital letter are significantly different from each other, assayed by one-way analysis of variance and followed by Duncan's multiple range test ($p < 0.05$).
[d] The sensitivity of enzyme-linked immunosorbent assay kits used in this study was <15.6 pg/mL.
[e] n.d. = not detectable.

2 + TNF-α)/(IL-4 + IL-5 + IL-10) (Th1/Th2) cytokine secretion ratios were significantly decreased by MP ($p < 0.05$), suggesting that MP has Th2 inclination and anti-inflammation potential in the absence of LPS. Table 4 shows the effects of MP on Th1/Th2 and pro-/anti-inflammatory cytokine secretion in the presence of LPS. The results showed that IL-4, IL-5, IL-10, IL-12, and TNF-α levels secreted by the MP-treated splenocytes significantly increased in a concentration-dependent manner ($p < 0.05$). IL-2 also significantly increased by MP ($p < 0.05$). Our results suggested that MP has strong immunostimulatory effects in the presence of LPS. However, (IL-2 + TNF-α)/(IL-4 + IL-5 + IL-10) (Th1/Th2) and TNF-α/IL-10 (pro-/anti-inflammatory) cytokine secretion ratios were not significantly influenced by MP ($p > 0.05$), suggesting that MP could not fully reverse anti-inflammation status in the presence of LPS.

Among cytokines, IL-10 that is produced in late stage inflammation by immune effector cells to inhibit the synthesis of other cytokines has been recognized as a Th2 and anti-inflammatory cytokine [23]. By contrast, the maximum secretion of proinflammatory cytokines such as TNF-α and IL-1β occurs at 12–48 hours when inflammation starts [24,25]. Therefore, cytokine secretion profiles can reflect the inflammation status of target cells. In this study, we found that SP and MP treatment increased IL-10 (an anti-inflammatory cytokine) production by murine primary splenocytes in the absence or presence of LPS in a concentration-dependent manner, suggesting solid anti-inflammation potential of SP and MP (Tables 1–4). Furthermore, SP significantly decreased Th1/Th2 cytokine secretion ratios in the absence or presence of LPS, but just slightly decreased pro-/anti-inflammatory cytokine secretion ratios by splenocytes in the presence of LPS (Tables 1 and 2). Our results suggested that SP may exert its anti-inflammatory ability via decreasing Th1/Th2 cytokine secretion ratios rather than directly inhibiting proinflammatory cytokine secretion by inflamed cells. Similarly, MP also significantly decreased Th1/Th2 cytokine secretion ratios in the absence of LPS (Table 3), but it could not significantly influence Th1/Th2 and pro-/anti-inflammatory cytokine secretion ratios by splenocytes in the presence of LPS (Table 4). Our results suggested that MP had a worse ability at anti-inflammation than SP. Taken together, our findings indicated that SP has better anti-inflammatory potential than

Table 2 – Effects of strawberry fruit polysaccharide on cytokine secretion by lipopolysaccharide (LPS)-stimulated splenocytes from BALB/c mice.

Cytokine levels (pg/mL)[a,b,c,d,e,f]	SP (μg/mL)			
	0	250	500	1000
IL-2	22 ± 1	25 ± 3	24 ± 5	22 ± 4
IL-4	n.d.	n.d.	n.d.	n.d.
IL-5	n.d.	n.d.	n.d.	n.d.
IL-10	118 ± 49^{B}	507 ± 86^{A}	668 ± 152^{A}	752 ± 182^{A}
IL-12	17 ± 1^{ABC}	14 ± 3^{BC}	14 ± 0^{BC}	24 ± 2^{A}
TNF-α	261 ± 114^{B}	552 ± 72^{A}	773 ± 113^{A}	648 ± 35^{A}
IL-2/IL-10 (pg/pg)	0.19 ± 0.09^{A}	0.05 ± 0.00^{B}	0.04 ± 0.00^{B}	0.03 ± 0.00^{B}
TNF-α/IL-10 (pg/pg)	2.10 ± 0.16	1.20 ± 0.29	1.6 ± 0.49	1.10 ± 0.29

[a] Data are mean ± SEM ($n = 6$ biological determinations).
[b] The cell density was 5×10^6 cells/mL.
[c] Data within the same row not sharing a common superscript capital letter are significantly different from each other, assayed by one-way analysis of variance and followed by Duncan's multiple range test ($p < 0.05$).
[d] The LPS concentration was 5 μg/mL.
[e] The sensitivity of enzyme-linked immunosorbent assay kits used in this study was <15.6 pg/mL.
[f] n.d. = not detectable.

Table 3 – Effects of mulberry fruit polysaccharide (MP) on cytokine secretion by murine primary splenocytes.

Cytokine levels (pg/mL)[a,b,c,d]	MP (μg/mL)			
	0	250	750	1250
IL-2	25 ± 5^{C}	17 ± 4^{C}	55 ± 6^{B}	316 ± 12^{A}
IL-4	1 ± 1^{C}	5 ± 3^{C}	31 ± 8^{B}	100 ± 16^{A}
IL-5	6 ± 3^{D}	58 ± 8^{C}	197 ± 20^{B}	737 ± 204^{A}
IL-10	14 ± 7^{C}	496 ± 70^{B}	575 ± 88^{AB}	749 ± 61^{A}
IL-12	16 ± 11^{B}	19 ± 7^{AB}	24 ± 8^{AB}	29 ± 6^{A}
TNF-α	106 ± 30^{C}	577 ± 53^{B}	732 ± 30^{A}	847 ± 11^{A}
(IL-2 + TNF-α)/(IL-4 + IL-5 + IL-10) (pg/pg)	6.10 ± 1.35^{A}	1.10 ± 0.08^{B}	1.10 ± 0.08^{B}	0.80 ± 0.12^{B}

[a] Data are mean ± SEM ($n = 6$ biological determinations).
[b] The cell density was 5×10^6 cells/mL.
[c] Data within the same row not sharing a common superscript capital letter are significantly different from each other, assayed by one-way analysis of variance and followed by Duncan's multiple range test ($p < 0.05$).
[d] The sensitivity of enzyme-linked immunosorbent assay kits used in this study was <15.6 pg/mL.

that of MP *in vitro*. Undoubtedly, our results suggest that SP and MP, particularly SP, isolated from fruit juices may be ingested daily to protect the body from mild inflammation via modulating Th1/Th2 cytokine secretion ratios *in vivo*. In our previous study, SP and MP treatment significantly decreased proinflammatory cytokines including IL-1β and IL-6 by mouse peritoneal macrophages ($p < 0.05$), whereas the anti-inflammatory cytokine IL-10 was markedly increased, suggesting that SP and MP have anti-inflammation potential via modulating pro-/anti-inflammatory cytokine secretion profiles [14]. These findings in the present study are identical to previously published reports.

In comparison to the effects of SP and MP on cytokine secretion, the differences between SP and MP were that MP markedly stimulated IL-2 (a T cell growth factor), IL-4 (B cell growth factor 1, BCGF1), and IL-5 (B cell growth factor 2, BCGF2) production, but SP did not (Tables 1–4). Our results indicated that in MP-treated cultures T- and B cells proliferated, but SP did not significantly stimulate T- and B cell proliferation (Fig. 1). However, active polysaccharides, such as SP and MP, may stimulate antigen-presenting cells (APCs) including B cells, macrophages, and dendritic cells that produce IL-10, IL-12, and TNF-α (Tables 1–4).

In our preliminary studies and other published studies, we found that LPS from 1 μg/mL to 100 μg/mL was recommended to treat primary cells for inducing inflammation *in vitro*. Therefore, we selected LPS at 5 μg/mL to induce mild inflammation in splenocytes but not severe inflammation. We have found that LPS at 5 μg/mL indeed induced inflammation in mouse peritoneal macrophages and secreted a large amount of TNF-α *in vitro* [14]. Splenocytes (for the most part of lymphocytes) in nature secrete less TNF-α levels than that by macrophages. Thus, LPS treatment alone at the indicated concentration of 5 μg/mL just slightly elevated TNF-α levels in splenocyte cultures (from 179 ± 80 pg/mL to 261 ± 114 pg/mL in Tables 1 and 2; from 106 ± 30 pg/mL to 250 ± 4 pg/mL in Tables 3 and 4). However, these data evidenced that LPS at 5 μg/mL induced mild inflammation in splenocytes but not severe inflammation.

3.3. *SP and MP treatment effects on anti- (Bcl-2) and proapoptotic (Bak) protein levels in murine primary splenocytes*

Changes in Bcl-2 family members including one of the anti-apoptotic family members (Bcl-2 protein) and one of the

Table 4 – Effects of mulberry fruit polysaccharide on cytokine secretion by lipopolysaccharide (LPS)-stimulated splenocytes from BALB/c mice.

Cytokine levels (pg/mL)[a,b,c,d,e]	MP (μg/mL)			
	0	250	750	1250
IL-2	22 ± 4^{C}	15 ± 6^{C}	84 ± 22^{B}	326 ± 56^{A}
IL-4	2 ± 1^{C}	6 ± 4^{C}	33 ± 6^{B}	88 ± 11^{A}
IL-5	9 ± 4^{C}	25 ± 8^{C}	199 ± 29^{B}	662 ± 198^{A}
IL-10	226 ± 16^{C}	618 ± 102^{AB}	699 ± 98^{AB}	787 ± 91^{A}
IL-12	8 ± 4^{B}	19 ± 7^{AB}	22 ± 7^{AB}	37 ± 4^{A}
TNF-α	250 ± 4^{C}	530 ± 73^{B}	749 ± 23^{A}	796 ± 33^{A}
(IL-2 + TNF-α)/(IL-4 + IL-5 + IL-10) (pg/pg)	1.20 ± 0.08	0.90 ± 0.12	1.00 ± 0.12	0.90 ± 0.20
TNF-α/IL-10 (pg/pg)	1.10 ± 0.08	0.90 ± 0.12	1.20 ± 0.16	1.10 ± 0.12

[a] Data are mean ± SEM ($n = 6$ biological determinations).
[b] The cell density was 5×10^6 cells/mL.
[c] Data within the same row not sharing a common superscript capital letter are significantly different from each other, assayed by one-way analysis of variance and followed by Duncan's multiple range test ($p < 0.05$).
[d] The LPS concentration was 5 μg/mL.
[e] The sensitivity of enzyme-linked immunosorbent assay kits used in this study was <15.6 pg/mL.

proapoptotic subfamily members (Bak protein) in target cells treated by SP or MP in the absence of LPS were determined using the Western blotting assay method to clarify the anti-apoptotic mechanisms of SP and MP on splenocytes. The results showed that SP treatment at the indicated concentration of 1000 μg/mL significantly inhibited Bcl-2 level in splenocytes compared to the control ($p < 0.05$), whereas SP treatment at the same concentration also slightly inhibited Bak level in the splenocytes ($p > 0.05$; Fig. 2). Further comparison with Bcl-2/Bak (anti-/proapoptotic) protein levels in the splenocytes exhibited that SP treatment at the indicated concentrations did not markedly affect Bcl-2/Bak (anti-/proapoptotic) protein ratios in the splenocytes ($p > 0.05$; Fig. 2). Our results suggested that SP treatment alone increased splenocyte proliferation (Fig. 1A), but not through modulating the Bcl-2/Bak (anti-/proapoptotic) pathway (Fig. 2). Moreover, we found that cell proliferation was not necessarily equal to antiapoptosis in murine primary splenocytes [21,22]. This work and these findings are identical to previously published reports.

Our results showed that MP treatment at the indicated concentrations did not significantly affect Bcl-2 and Bak levels in the splenocytes compared to the control ($p > 0.05$; Fig. 3). Importantly, further comparison of Bcl-2/Bak (anti-/proapoptotic) protein ratios in the splenocytes exhibited that MP treatment at the indicated high concentration of 1250 μg/mL significantly increased the Bcl-2/Bak (anti-/proapoptotic) protein expression ratio, suggesting that appropriately higher MP concentrations markedly inhibited apoptosis in murine primary splenocytes. We theorize that low MP concentration could still not reverse spontaneous apoptosis in murine primary splenocytes due to low zone immune response tolerance. Our results suggest that low and high MP concentrations exerted differential effects on mitochondrial Bcl-2/Bak (anti-/proapoptotic) protein expression ratios in splenocytes. Differential protective effects of dietary polyphenols quercetin, resveratrol, rutin, and epigalloctechin gallate against mitochondrial dysfunction induced by indomethacin in Caco-2 cells are reported [26]. Polysaccharide of *Ulva lactuca* was also found to stabilize mitochondrial and microsomal function in D-galactosamine induced hepatitis in rats [27]. Our results further suggest that MP at appropriate concentrations may stabilize mitochondrial function in primary splenocytes via modulating mitochondrial Bcl-2/Bak (anti-/proapoptotic) protein expression. Our results further suggest that the proliferation of splenocytes exerted by MP (Fig. 1B) might through inhibiting apoptosis (Fig. 3). When cytokine secretion profiles correlate with proliferative and antiapoptotic effects of splenocytes treated by SP and MP, it could be assumed that MP raised IL-2 (T cell growth factor), IL-4 (BCGF1), and IL-5 (BCGF2) production by the cells (Tables 3 and 4), sequentially stimulating the proliferation of both splenic T and/or B cells (Fig. 1B), and consequently inhibiting their apoptosis (Fig. 3). However, antiapoptotic mechanisms of MP should be studied further in the future.

It was found that polysaccharides isolated from mulberry leaves, MPS-1 (25 kDa) and MPS-2 (61 kDa), were composed of Sor, Ara, Xyl, and Glc, and Rha, Ara, Xyl, Glc, Gal, and Man, respectively [28]. SP and MP carbohydrate and protein constituent ratios were 43%:57% (w/w) and 28.4%:71.6% (w/w),

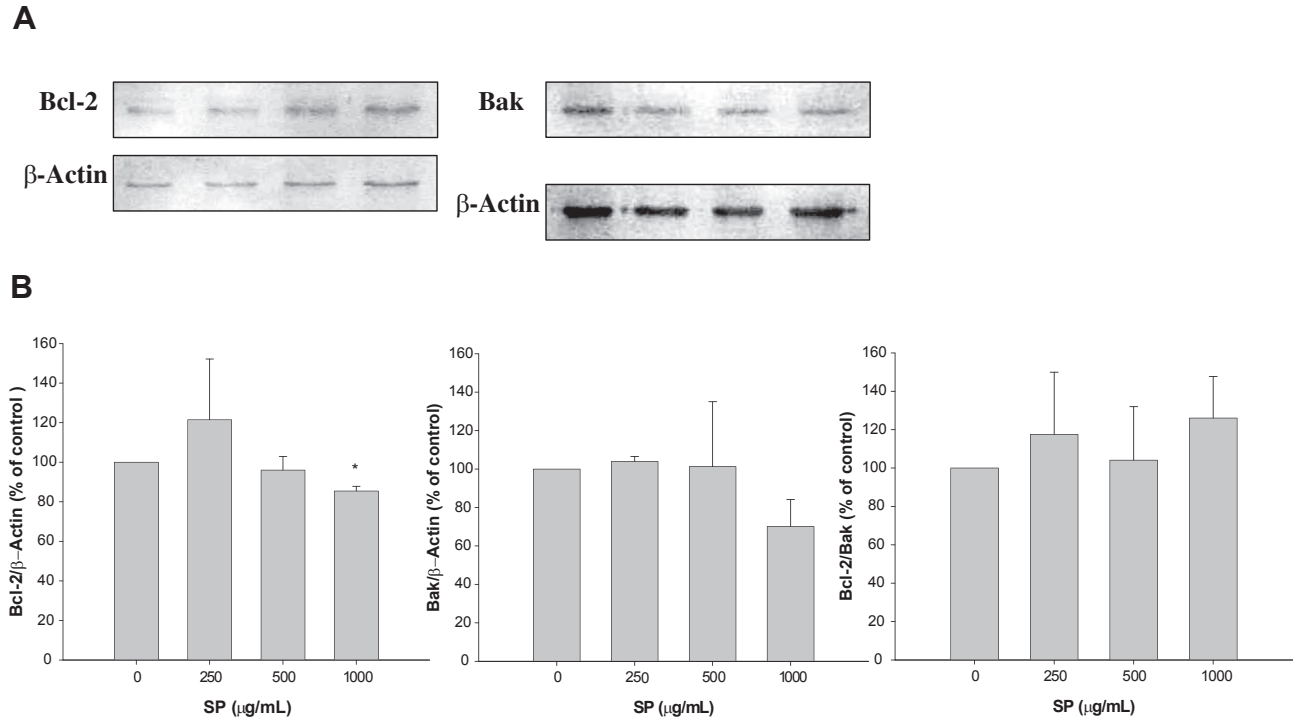

Fig. 2 – Effects of strawberry fruit polysaccharide (SP) on Bcl-2 and Bak protein expression in splenocytes from BALB/c mice. (A) Western blots of Bcl-2, Bak, and β-Actin proteins. (B) Densitometric analyses of relative Bcl-2, Bak, and Bcl-2/Bak protein levels to β-Actin. Data are mean ± SEM ($n = 2$ biological determinations). Asterisk (*) means significantly different among different treatments and controls (reference), analyzed by one-way analysis of variance, followed by Dunnett's *t* test ($p < 0.05$).

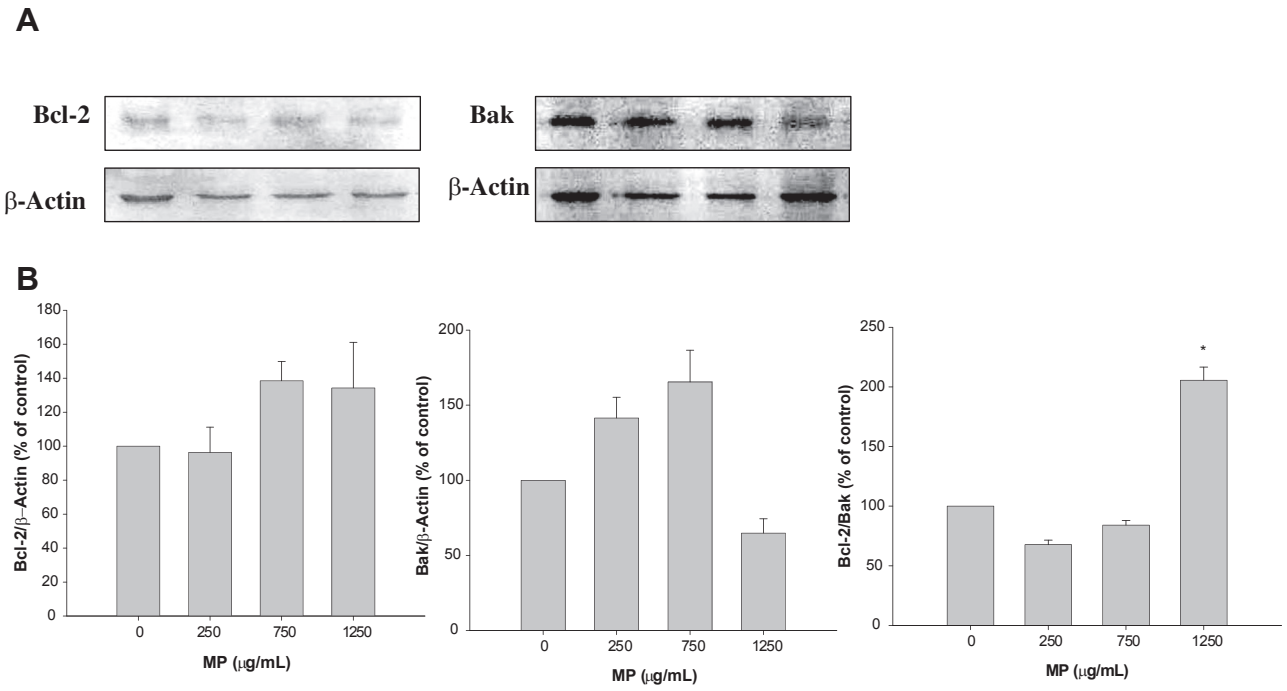

Fig. 3 – Effects of mulberry fruit polysaccharide (MP) on Bcl-2 and Bak protein expression in splenocytes from BALB/c mice. (A) Western blots of Bcl-2, Bak, and β-Actin proteins. (B) Densitometric analyses of relative Bcl-2, Bak, and Bcl-2/Bak protein levels to β-Actin. Data are mean ± SEM ($n = 2$ biological determinations). Asterisk (*) means significantly different among different treatments and controls (reference), analyzed by one-way analysis of variance, followed by Dunnett's t test ($p < 0.05$).

respectively, suggesting that both SP and MP may be glycoproteins, and in addition may well tolerate gastroenteric digestion and exert their effects *in vivo* through Peyer's patches that are aggregated lymphoid nodules in the small intestine [14]. The present study has suggested that SP has better anti-inflammation potential (Tables 1–4), whereas MP has better cell proliferation and antiapoptotic potential *in vitro* (Figs. 1B and 3). However, more data concerning the characteristics, anti-inflammatory, and antiapoptotic mechanisms of SP and MP should be accumulated to further clarify the physiological potential of fruit polysaccharides in the future [29].

Although the present study has some advantages, there are some limitations. The representative marker cytokine for Th1 is IFN-γ, and for Th2 is IL-4. Unfortunately, in this study IFN-γ was not determined due to its low secretion levels in nature; therefore, we selected IL-2 as a Th1 indicator. However, IL-2 is a common T cell growth factor, although it is primarily produced by effector T cells (i.e., Th1). Moreover, IL-4 secretion levels by the splenocytes treated with SP were too low to be detectable (Tables 1 and 2); therefore, IL-10 was selected as a Th2 indicator, although IL-10 and TNF-α are mainly produced by macrophages or APCs. Even though mouse primary splenocytes may contain approximately 10% APCs and other cells because primary splenocytes are mainly composed of 41.54% B cells and 47.11% T cells [22], using the single ratio of IL-2/IL-10 to indicate the status of Th1/Th2 or of TNF-α/IL-10 to determine pro-/anti-inflammatory condition by splenocytes might not be completely correct. Experiments using B or T cell depleted splenocytes and macrophages should be repeated in the future to prove our hypothesis. In addition, the concentrations of SP and MP used at 250–1000 μg/mL required to demonstrate effects on cultured splenocytes are high, although the samples are still crude polysaccharides. Furthermore, strawberry and mulberry fruits contain abundant ellagitannins and flavonoids that are not ethanol/water soluble. In fact, we found trace amounts of flavonoids, including rutin and quercetin, in MP that might contribute to their effects on the splenocytes. Our previous results have indicated that the maximum absorption peak of SP and MP appeared at 240 nm with a small shoulder around 280–310 nm, suggesting that SP and MP might be glycoproteins [14]. However, SP and MP should be further purified and characterized in the future.

4. Conclusions

This study isolated polysaccharides from strawberry and mulberry fruits to assess their anti-inflammatory and antiapoptotic activities using murine primary splenocytes in the absence or presence of LPS. Our results provide evidence that SP and MP treatment at appropriate concentrations significantly increased the proliferation of splenocytes. SP and MP treatment in the absence of LPS, and SP treatment in the presence of LPS markedly decreased IL-2/IL-10 (Th1/Th2) ratios, and SP in the presence of LPS slightly decreased TNF-α/IL-10 (pro-/anti-inflammatory) cytokine ratios by splenocytes, suggesting that SP has better anti-inflammation potential than MP. However, MP treatment at an appropriate concentration in the absence of LPS showed antiapoptotic activity via modulating pro- (Bak) and

antiapoptotic (Bcl-2) protein expression ratios, suggesting that MP may protect primary immune cells from apoptotic cell death. Overall, our findings suggest that SP has better anti-inflammation potential, whereas MP has better cell proliferation and antiapoptotic potential *in vitro*.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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